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Biotype-specific expression of dsRNA in the sweetpotato whitefly

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Abstract

This study was conducted to evaluate the effect of two different biotypes of the sweetpotato whitefly, *Bemisia tabaci* (Gennadius), on the induction of squash silverleaf (SSL), and to determine if double-stranded RNA (dsRNA) occurs in geographically remote populations of the two biotypes. Recently collected B-biotype whiteflies from Florida, Arizona, Mississippi, and Texas (SPW-B) all contained a 7.0 kb dsRNA molecule. Kb dsRNA molecule. Laboratory colonies of A-biotype whiteflies that were originally collected in 1981 from cotton in Arizona and California did not contain the 7.0 Kb dsRNA. When the two biotypes were compared only the SPW-B induced rapid onset, grade 5, SSL. DsRNA similar to that found in adult SPW-B was concentrated in whitefly nymphs, but host plant leaf tissue did not contain any consistent dsRNA molecules. SPW-A only induced low-grade SSL and progeny of SPW-A that were fed on pumpkin plants displaying SSL did not acquire the ability to express dsRNA or induce SSL. Our data suggest that dsRNA is not directly involved in the induction of SSL and that SSL is a host-specific response to a feeding injury induced by B-biotype whiteflies. The origin and source of the 7.0 Kb dsRNA molecule remains enigmatic but its expression is constant in the whitefly biotype that is responsible for the induction of SSL and several other plant disorders in the U.S.

Introduction

In 1987–1988 squash silverleaf (SSL) and tomato irregular ripening (IRR) caused substantial economic losses in the state of Florida. Initial assessment of these disorders suggested environmental factors or poor crop management. However, by 1989 both SSL and IRR were attributed to the presence of elevated populations of the sweetpotato whitefly (SPW), *Bemisia tabaci* (Gennadius) (Maynard & Cantliffe, 1989).

Following the association of SPW with SSL, two divergent hypotheses were advanced to explain this disorder. Bharathan *et al.* (1990) reported two double-stranded RNAs (dsRNA; 4.6 and 4.2 kilobase pairs, Kb) in leaf extracts from symptomatic plants and implicated this foreign nucleic acid as the causal agent of whitefly-induced SSL. Yokomi *et al.* (1990) also discovered dsRNA (4.4 and 4.0 $\times$ 10^6 mol wt), but this nucleic acid was only found in the SPW; dsRNA was not found in symptomatic leaves. Yokomi *et al.* (1990) suggested that SSL was caused by a translocated toxicogenic factor associated with whitefly feeding, and speculated that the dsRNA from the SPW might be the result of an insect virus.

By 1991, whitefly-induced SSL, IRR, and discoloration disorders in cole crops had been reported throughout the southern United States

(Brown *et al.*, 1992; Cohen *et al.*, 1992; Perring *et al.*, 1991), and SSL had been documented in Hawaii, Japan, and parts of the Caribbean basin (Costa *et al.*, 1993; Nairiko, 1991; Segarra-Carmona *et al.*, 1990). Physiological and biological data suggest that the whitefly responsible for these disorders (tentatively referred to as the B-biotype) was introduced into the United States (Culotta, 1991), but the genetic distance between this new whitefly and the whitefly that was prevalent before 1986 (A-biotype) is unknown (Byrne & Miller, 1990; Campbell, 1993; Costa & Brown, 1992; Perring *et al.*, 1992). In this study, we tested the hypothesis that two biotypes of whitefly exist by comparing the effects of two different populations on the induction of SSL and by examining the nucleic acid content of a variety of host plants and whiteflies for dsRNA. All of the B-biotype SPW induced SSL and contained the dsRNA reported by Yokomi *et al.* (1990). We found that the expression of dsRNA and the ability to induce SSL were heritable traits that were not acquired by the original A-biotype whiteflies after feeding from plants infested with the B-biotype SPW. All of our data suggest that the induction of SSL is not dependent on the expression of dsRNA in cucurbits.

Materials and methods

Insects and plants. Colonies of SPW were maintained in an evaporatively cooled greenhouse on eggplant, *Solanum melogena* L. cv. 'Florida market 10'; squash, *Cucurbit pepo* L. cv. 'Dixie'; melon, *C. melo* cv. 'Edisto'; tomato, *Lysopersicon esculentum* M. cv. 'Sunny' (all from Asgrow Seed Co., Kalamazoo, MI); and pumpkin, *C. pepo* L. cv. 'Small sugar' (W. Atlee Burpee & Co., Warminster, PA). Our endemic sweetpotato whitefly population is referred to as SPW-B; however, this does not denote geographic origin or current isolation of this race. SPW-B and different host plants were collected from several locations throughout central Florida, including greenhouse-grown ornamentals and vegetable crops where pesticide application was heavy, as well as small greenhouse and urban settings where selective pressure was reduced. Additional samples of whitefly-infested material were imported from areas in which silverleaf and sweetpotato whitefly had become problematic.

We analyzed two distinct SPW populations from Arizona. Arizona B-biotype SPWs were originally collected from poinsettia plants in 1989. Arizona A-biotype SPWs were originally collected from cotton in 1981. Both Arizona biotypes were provided courtesy of Dr. Judith K. Brown, Univ. of Arizona, Tucson, AZ. *Bemisia tabaci* from Mississippi (courtesy of Dr. Michael Smith, USDA-ARS, Stoneville, MS) and Texas (courtesy of Dr. Mani Skaria, Texas A&I, Weslaco, TX) were also analyzed.

In 1990, we initiated a whitefly colony using SPW originally collected in 1981 from cotton in California's Imperial Valley. These A-biotype SPWs (documented as IV-81 in Cohen *et al.*, 1992) were provided courtesy of Dr. James Duffus, USDA-ARS, Salinas, CA. These whiteflies were maintained in a separate greenhouse within 0.16-m^3 isolation cages constructed of splined aluminum frames covered with monofilament nylon screen (300-μm mesh Nitex, Aquatic Eco-Systems Inc., Apopka, FL) and clear acrylic. Access through sleeved openings and constant monitoring prevented contamination by endemic SPW-B. This colony was maintained on pumpkin and squash which were closely monitored for SSL and the expression of dsRNA. We will refer to these A-biotype insects as SPW-A. Esterase isozyme assays and banding patterns reported by Costa & Brown (1992) and Liu *et al.* (1992) have shown that our SPW-A and SPW-B colonies express esterase patterns indicative of A- and B-biotype SPWs respectively (unpubl. data).

Trialeurodes vaporariorum (Westwood), *T. variablis* (Quaintance), and *T. abutilonea* (Haldeman) were analyzed for dsRNA as well. Unless specified, all experiments assessing whitefly-induced SSL were performed using *C. pepo* L. cv. 'Small sugar' pumpkin, or cv. 'Dixie' squash. The seasonal and sporadic appearance of a variety of dsRNA bands from these host plants required that we evaluate the nucleic acid content of the

saprophytic molds associated with whitefly honeydew and necrotic leaf tissue. Leaves colonized by whiteflies, mold that grew on whitefly honeydew, and individual whiteflies were homogenized in sterile water using an autoclaved tissue homogenizer. This crude homogenate was used to prepare spread plates on Sauboraud's dextrose agar (SDA) and potato dextrose agar (PDA). Plates were incubated until confluent with a mixture of molds and then the entire plate assayed for dsRNA.

SSL and dsRNA. To compare the effect of SPW-A and SPW-B on the expression of SSL and dsRNA, previously described 0.16-m^3 plexiglass isolation cages were set up in an evaporatively cooled greenhouse under ambient light. Two hundred adult SPW-A were removed from the colony and placed in an isolation cage with three 1-gallon pots containing 3-week-old pumpkin plants. Two hundred SPW-B were introduced into an identical cage, and a third cage was set up without whiteflies as a control. Plants were harvested 25 days after the SPW were introduced and the tissue was analyzed for SSL and dsRNA.

To evaluate the effect of high intensity light on the induction of SSL, six cages were set up in the greenhouse under four VHO fluorescent bulbs on a 16:8 photocycle. Pumpkin plants were preconditioned as described by Cohen *et al.* (1992) and light measurements were taken with a Trilux footcandle meter (P. Gossen and Company, West Germany). Light intensity varied from 2000 lux at the base of the cage to 28,000 lux at the top. Each cage contained six 3-week-old pumpkin plants in three 1-gallon pots. Two cages were maintained as controls, two were inoculated with 200 SPW-A, and two were inoculated with 200 SPW-B. After three weeks the plants were harvested, the SSL symptoms were graded, and the SPW immatures were counted. Leaves bearing SPW were separated from the leaves displaying silvering and analyzed for dsRNA.

The extraction, purification, and analysis of dsRNA were carried out as previously reported (Yokomi *et al.*, 1990). Extraction and purification protocols were also modified according to methods reported by Bharathan *et al.* (1990), Dodds *et al.* (1984), and Jordan & Dodds (1985), without any significant change in the results. Total RNA extractions were carried out according to Chomczynski & Sacchi (1987), as modified by Puissant & Houdebine (1990). Lithium chloride extraction was necessary with samples of adult whiteflies and leaf tissue infested with whitefly nymphs to remove residual carbohydrate in the nucleic acid pellet. Total RNA extracts were analyzed on 4-mm, 1% horizontal agarose gels in 1X Tris-Borate-EDTA buffer, pH 8.3, at 40 mA for 4 h, and stained with ethidium bromide.

Adult SPW were also extracted using guanidinium isothiocyanate and the RNA was electrophoresed in denaturing glyoxal-DMSO gels, or purified using Oligo-dT chromatography as described by Sambrook *et al.* (1989). One- to two-g samples of SPW-B were subjected to virus isolation strategies based on methods reported for insect single- and double-stranded RNA viruses (D'Arcy *et al.*, 1981; Jousset *et al.*, 1977; Rybicki & von Wechmar, 1982). Following extraction, differential centrifugation, and rate zonal centrifugation, sucrose gradients were scanned and fractions showing high A_{260} and high $A_{260:280}$ ratios were pelleted. Some fractions were resuspended, negatively stained, and viewed on formvar-coated grids; some were fixed, embedded, sectioned, stained, and then viewed in a Philips 201 transmission electron microscope.

Acquisition and transmission. To test whether the SPW-A could acquire the ability to express dsRNA, or induce SSL, adult SPW were confined in small clip cages on pumpkin leaves displaying SSL, or on pumpkin and eggplant leaves colonized by SPW-B. Paired tests using SPW-A and SPW-B were conducted to ensure that adult mortality was not due to the acquisition of an insect virus, inadequate nutrition, or caging. Fifty female and five male SPW were placed in each cage. Cages consisted of a 21-cm^3 clear cylindrical acrylic vial fitted with a nylon screen (300-μm mesh) at both ends. A spring-loaded hair clip held the leaf blade sandwiched between the lower screen and a foam-lined support. This allowed

the adult insects to feed and oviposit through the mesh, but kept the SPW biotypes separated. After five days, surviving insects were counted and transferred to isolation cages containing 3-week-old pumpkin plants. The progeny of these insects were monitored for a minimum of four months. Leaves bearing nymphs were extracted and analyzed for dsRNA, and pumpkin plants were compared to plants infested with SPW-B for the induction of SSL.

Results

SSL–Double-stranded RNA. Table 1 shows a list of host plants and whitefly species analyzed for SSL and dsRNA. The expression of squash silverleaf was limited to members of the Cucurbitaceae. All host plant tissue harboring SPW-B nymphs contained dsRNA bands similar to those reported by Yokomi *et al.* (1990). Adult whiteflies and leaves infested with B-biotype whiteflies from Arizona were dsRNA-positive but the Arizona A-biotype whiteflies were dsRNA-negative. Leaf material bearing SPW immatures, and adult SPW from Texas and Mississippi, were dsRNA-positive. The SPW-A from California and other species of whitefly did not contain the dsRNA reported by Yokomi *et al.* (1990). A myriad of both larger and smaller molecular weight dsRNA bands were isolated from plants infested with either SPW-A or SPW-B. These dsRNA appeared seasonally and sporadically. Some were traced to the presence of fungi associated with whiteflies, sooty mold, and necrotic leaf tissue (Fig. 1).

In the fall of 1990, in the first experiments to compare the two biotypes, pumpkin plants caged with SPW-B developed grade 5 SSL within 14 days. The leaves colonized by SPW-B nymphs were dsRNA-positive but the silvered leaf tissue was dsRNA-negative. After 20 days, pumpkin plants caged with SPW-A developed weak veinal chlorosis (grade 1 SSL), but the adults and the leaf tissue they colonized were dsRNA-negative. Table 2 and Figure 2 show the distribution of whitefly nymphs on the colonized pumpkin

Table 1. Host plants and whiteflies analyzed for dsRNA and SSL symptoms

Host plants and whitefly species	SSL	dsRNA[d]
Cucurbitaceae spp.		
Cucurbita pepo L. cv. 'Dixie'	+[e]	+
Curcurbita pepo L. cv. 'Small Sugar'	+	+
Cucurbita pepo L. cv. Senator[a]	+	+
Cucurbita foetidisma H.B.K. (Buffalo gourd)[a]	–[f]	+
Cucumis melo L. (Japanese muskmelon)[a]	–	+
Cucumis sativus L. (European Cucumber)[b]	–	+
Cucurbita melo L. (Cantaloupe)	–	+
Non-Cucurbitaceae		
Solanum melogena L. (Eggplant)	–	+
Lycopersicon esculentum M. (Tomato)	–	+
Capsicum annuum L. (Bell pepper)[a]	–	+
Capsicum frutescens L. (Jalapeno pepper)[a]	–	+
Helioanthus annus L. (Sunflower)[a]	–	+
Origanum vulgare L. (Oregano)[a]	–	+
Hibiscus cannabinus L. (Kenaf)	–	+
Hibiscus esculentum L. (Okra)	–	+
Psophocarpus tetragonolobus L. (Wing bean)[a]	–	+
Whitefly species		
Bemisia tabaci G. (SPW-B)	+[g]	+
Bemisia tabaci G. (SPW-A)	+/–[h]	–
Bemisia tabaci G. (Arizona B-biotype)	+	+
Bemisia tabaci G. (Arizona A-biotype)	+/–	–
Bemisia tabaci G. (Texas B-biotype)	+	+
Bemisia tabaci G. (Mississippi B-biotype)	n.d.	+
Trialeurodes vaporariorum W. (Greenhouse whitefly)[b]	n.d.	–
Trialeurodes abutilonea H. (Bandedwinged whitefly)[c]	n.d.	–
Trialeurodes variablis Q. (Papaya whitefly)[c]	n.d.	–

[a] Courtesy of Dr. F. Petit & Ms. M. Knopp, Epcot Center, The Land, Orlando, Florida. [b] Courtesy of Mr. P. Smits, Burnac Produce, Port St. Lucie, Florida. [c] Courtesy of Dr. L. Osborne, IFAS, & Dr. K. Hoelmer, USDA-ARS, Apopka, Florida. [d] + = detectable; – = not detectable dsRNA similar to those reported by Yokomi *et al.* (1990). [e] Expressed SSL. [f] Does not express SSL. [g] Able to induce SSL. [h] Able to induce low-grade silvering. n.d.; not done.

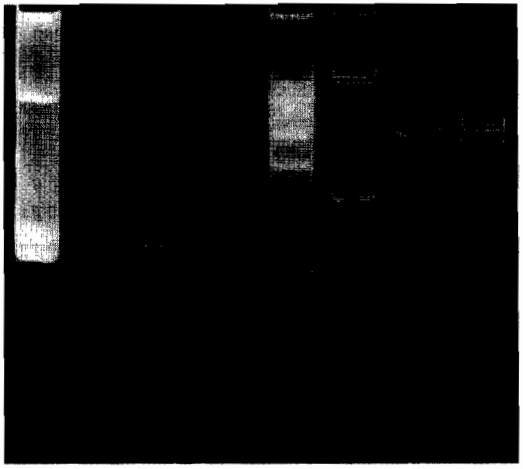

Fig. 1. Fungal dsRNAs seasonally present in the SPW-SSL system. Lanes 1–6 are dsRNA from SDA and PDA spread plates inoculated with homogenized whiteflies. Lanes 7 and 8 are from SDA-spread plates inoculated with homogenates of pumpkin leaf tissue contaminated with saprophytic mold.

plants, the dsRNA extracted from colonized leaves, and the location within the plants of the expression of SSL. In this experiment and all subsequent analyses, we were unable to detect the dsRNA reported by Bharathan *et al.* (1990) in the silvered leaf tissue (SSL, Table 1). Regardless of the extraction technique, the only constant dsRNA was associated with the SPW-B. During the summer months when photoperiod and greenhouse temperature were increased, the SPW-A were able to induce grade 2–3 SSL in 'Small sugar' pumpkin, but the leaf tissue and adult whiteflies were always dsRNA-negative. By the fall of 1991, the SPW-A were again only able to induce grade 1 SSL. Contrary to the report by Cohen *et al.* (1992) high-intensity, long-duration light failed to increase the ability of the SPW-A to induce SSL in 'Small sugar' pumpkin. After 21 days the 12 pumpkin plants colonized with 200 SPW-B adults were infested with 12,187 nymphs and displayed grade 5 SSL. The 12 plants colonized with 200 SPW-A had 8,686 nymphs and grade 0–1 SSL. Figure 3 shows the results of total RNA extraction. In these experiments, the dsRNA was also associated with SPW-B colonized leaf tissue and the adult SPW-B. When compared to double-stranded DNA and single-stranded RNA (ssRNA), the SPW-B dsRNA was estimated to contain 7.0 Kb. Detectable levels of dsRNA were consistently encountered from 50-mg samples of SPW-B adults and as few as 300 late-instar nymphs. This level of expression has remained constant within this population for the last two years.

The 7.0 Kb SPW-B dsRNA could not be visualized using ethidium bromide or acridine orange following denaturing agarose electrophoresis. Undenatured dsRNA from SPW-B adults and denatured controls (0.24–9.5 Kb single-stranded RNA and a 7.5 Kb poly A-tailed RNA, BRL, Gaithersburg, MD) yielded visible bands. The denaturing conditions appeared to reduce the SPW-B dsRNA to fragments that were too small, or too low in concentration, to detect. When both the wash buffer and the eluant from Oligo-dT columns were visualized using 7% PAGE, it appeared that 60–70% of the dsRNA was retained by the Oligo-dT column (% estimated by ethidium bromide staining).

Although extracts of SPW-B contained fractions with high A_{260} values and $A_{260:280}$ ratios between 1.5 and 1.8, no virus particle has been visualized in any negatively stained or thin-sectioned preparation from any extraction protocol used to date.

Acquisition and transmission. SPW mortality in the clip cages was equal regardless of the SPW biotype and whether they fed on silvered leaf tissue, other SPW-B colonized leaf tissue, or healthy controls. Three successive experiments failed to produce a SPW-A population capable of inducing SSL or expressing the 7.0 Kb dsRNA.

Discussion

The results of this research indicate that SSL is a cultivar–specific response to feeding by the SPW-B. Our data show that the SPW-B is unique in both its ability to induce rapid-onset, grade 5

Table 2. Analysis of *Bemisia tabaci*-colonized pumpkin plants

Plant sample number		# of leaves/grade of symptoms	Wet weight weight (g)	Number of nymphs/instar	dsRNA
SPW-B	1a	9 CS[a]	10.1	2393 1–4th	+
	1b	11 2–5 SSL[b]	24.4	90 1–2nd	–
	2a	7 CS	6.3	915 1–4th	+
	2b	12 2–4 SSL	9.9	4 1–2nd	–
	3a	8 CS	14.1	541 1–4th	+
	3b	9 2–5 SSL	24.0	9 1–2nd	–
Totals		56	88.8	3952	
SPW-A	4a	8 CS	8.1	2675 1–4th	
	4b	12 0–1 SSL	16.0	273 1–2nd	–
	5a	8 CS	17.0	1494 1–4th	–
	5b	17 0–1 SSL	22.6	234 1–2nd	–
	6a	6 CS	5.6	1434 1–4th	–
	6b	9 0–1 SSL	9.3	456 1–2nd	–
Totals		60	78.6	6566	

Distribution of whitefly nymphs and SSL symptoms when 200 SPW-A and 200 SPW-B adults were introduced into adjacent isolation cages containing 3-week-old pumpkin plants. Numbers 1–6 are replicates that have been divided into 2 subsets consisting of CS[a]: leaves containing nymphs and the associated chlorotic spots and, SSL[b]: leaves showing the indicated grade of squash silverleaf. The varied level of leaf silvering has been graded on a scale of 0 to 5, with 0 representing no silvering, and 5 indicating silvering of the entire upper leaf surface (Paris *et al.*, 1987).

SSL and the presence of the 7.0 Kb dsRNA. Under greenhouse conditions, SPW-A induced low-level SSL, and this biotype did not contain dsRNA.

The only consistent dsRNA detected in our study was the 7.0 Kb band associated with SPW-B. No other dsRNAs appeared consistently in any host plant or insect analyzed. Whiteflies from geographically remote areas also contained the 7.0 Kb dsRNA. Our data indicate that this dsRNA is a consistent characteristic of B-biotype SPWs. The absence of dsRNA in IV-90 SPW (B-biotypes) reported by Cohen *et al.* (1992) was due to small sample size (H. Y. Liu, pers. comm.).

DsRNA analysis is a routine yet sensitive technique that can be performed in most modestly equipped laboratories. DsRNA analysis reveals the genomic nucleic acid of dsRNA viruses and the transient intermediates that exist when ssRNA viruses replicate (Valverde, 1990). Many plant viruses are vectored by SPWs (Duffus & Flock, 1982; Brown & Bird, 1992), and a new gemini virus has been characterized that is unique to the B-biotype SPW (Abouzid & Hiebert, 1991).

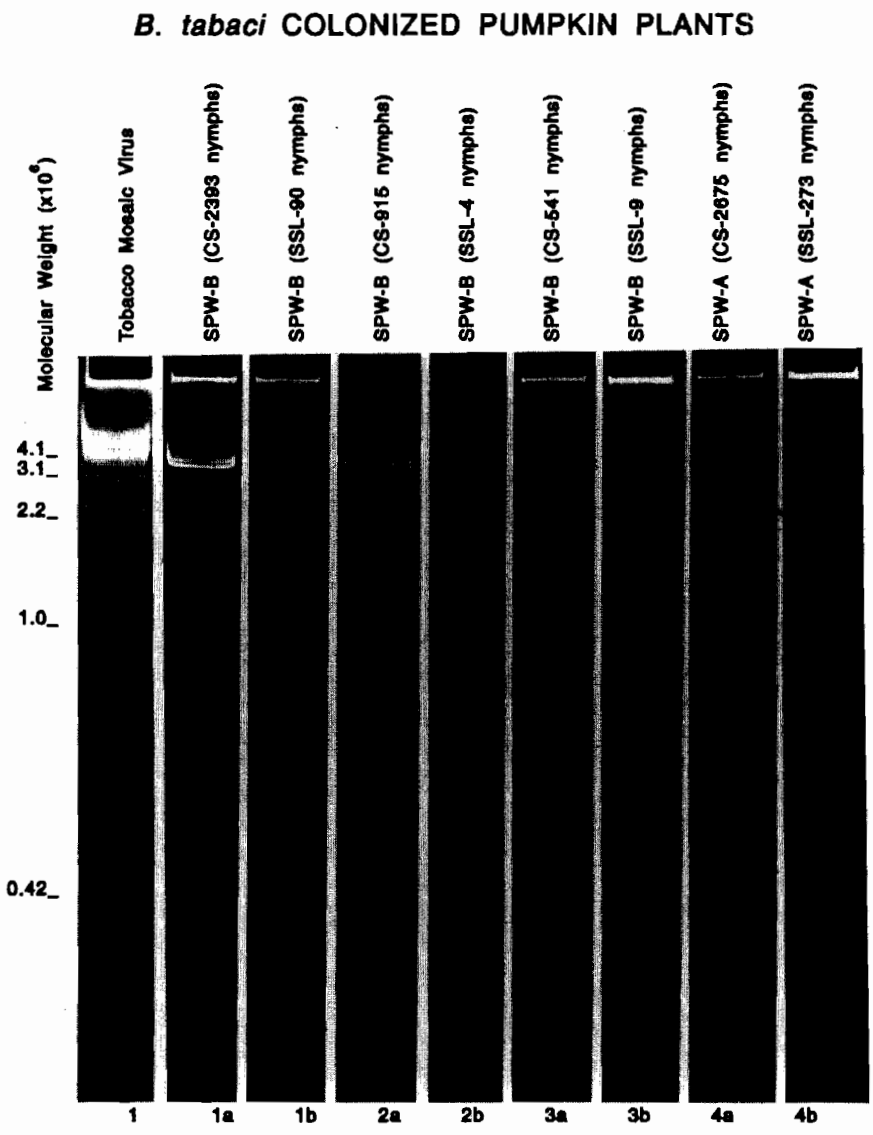

Fig. 2. Analyses of the plants documented in Table 2. Extracts were analyzed on 6% PAGE stained with ethidium bromide. Lane 1 is tobacco mosaic virus; lanes 1a–4b correspond to samples in Table 1. Lanes contain 1/3 of the dsRNA extracted from each sample.

Although Bharathan *et al.* (1990, 1992) have reported the association of two dsRNAs (4.6 and 4.2 Kb) with the induction of SSL, we have been unable to consistently detect any dsRNA in cucurbits expressing SSL. Numerous dsRNAs appear sporadically in samples of plant tissues colonized by both SPW biotypes, but the only consistent dsRNA is the 7.0 Kb molecule associated with the SPW-B. Our evidence suggests that dsRNA is not directly involved in the induction of SSL. The refractory nature of SSL in the absence of the SPW (Yokomi *et al.*, 1990) and the

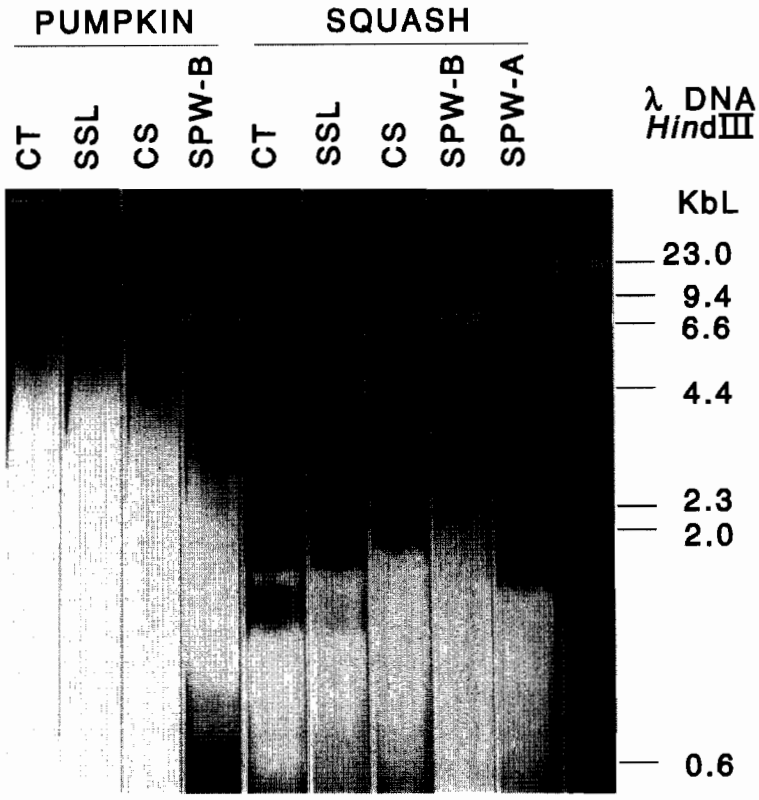

Fig. 3. Total RNA from 'Small sugar' pumpkin, 'Dixie' squash, and adult SPW. From left to right, samples contained the following: 1 g of control pumpkin (CT), silvered pumpkin (SSL), pumpkin infested with SPW-B nymphs (CS), 100 mg of adult SPW-B, 1 g of control squash, silvered squash, squash infested with SPW-B nymphs, 200 mg of adult SPW-B, and 200 mg of SPW-A. All lanes contain 1/3 of the RNA extracted from each sample. Standard lane contains 1 μg of *Hind* III cut Lambda DNA (BRL, Gaithersburg, MD).

anatomy and physiology of SSL (Paris *et al.*, 1987; Burger *et al.*, 1988; Jimenez *et al.*, 1993) indicate that this disorder is a host–specific response to B-biotype SPW feeding.

Some insect viruses use plants as circulative nonpersistent vectors. Several members of the *Picornaviridae* and the *Nodaviridae* infect insects and contain genomic RNA whose replication would result in dsRNA similar to that found in SPW-B (Matthews, 1982; Scotti *et al.*, 1981; Williamson *et al.*, 1988). Some of these viruses circulate nonpersistently in their plant hosts, and others cause lethal paralytic disorders in their insect hosts (D'Arcy *et al.*, 1981; Gildow & D'Arcy, 1988; Harrap & Payne, 1979; Ofori & Francki, 1985; Scotti *et al.*, 1983; Selling *et al.*, 1990). The presence of Oligo-dT binding poly-A tracts, the fragmentation of the dsRNA under denaturing conditions, and the size of the dsRNA would suggest that the B-biotype SPW might contain a picorna-like virus replicating through transient replicative intermediates, but we have not yet been able to isolate virus particles. Furthermore, we do not see seasonal fluctuations in the dsRNA levels, or elevated mortality in laboratory cultures of these insects. The inability of the SPW-A to acquire dsRNA suggests that neither pumpkin nor eggplant are adequate vectors of a putative virus, or that the dsRNA is only acquired through direct inheritance. Transcription and cloning of

the 7.0 Kb dsRNA are under way as a means of determining the source of this nucleic acid in the B-biotype SPW.

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